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13. ABSTRACT (Maximum 200 Words)

These studies were designed to improve the outcome of radioimmunotherapy (RIT) in prostate adenocarcinoma by the inclusion of vasoactive peptides in the RIT protocol. To date three peptides able to modify vascular permeability (VP) were tested. Cytotoxicity studies indicated dose-dependent changes in cell metabolic activities after treatment with two agonists interacting with CD88 (C5aAP peptides); whereas one peptide interactive with a formyl peptide receptor-like 1 did not seem to have any effect on the growth of these cells in vitro. In vivo results confirmed that at least two of these peptides significantly augmented RIT with ^{131}ICC49. The principal reason for improvements was identified as the increased tumor uptake of the radiotracer. However, there is also an indication that the generation of reactive oxygen species plays a significant role. The effect of the C5aAP peptide N2 containing an additional GCG sequence at the NH_2 terminus does not impair this peptides interactions with CD88. The N2's effect on VP and tumor uptake is virtually identical to the effects of the N1 peptide. The N2, however, considerably reduces the radiosensitivity of LNCaP, PC3 and DU145 cells in vitro. The in vivo studies of N2 with and without thiol group modification are in progress.

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PROGRESS REPORT

Introduction

The efficacy of radioimmunotherapy (RIT) in solid tumors depends on numerous factors related to the characteristics of the antibody and the radionuclide, and the tumor physiology such as the tumor heterogeneity, size, antigen distribution, radiosensitivity, microvessel perfusion, and vascular permeability, among others. It is apparent that a success of RIT in solid tumors will require a combination of therapeutic strategies to address simultaneously problems related to tumor physiology as well as the carrier and the type of radioisotopes.

The complement derived inflammatory mediator C5a brings on broad anaphylactic and chemotactic responses, including increased vascular permeability, changes in adhesiveness, smooth muscle contraction, and chemotactic migration of a number of cells. These biological activities are mediated through its binding to the C5a receptor (C5aR; CD88). C5a-derived small agonist peptides (C5aAP) from C-terminal region behave as full agonist, but with reduced potency. One analog, YSFKPMPLaR, expressed between 2 and 10% of full C5a activity in increasing vascular permeability and it was stable in the presence of mouse and human serum carboxypeptidases. Our studies showed that combination RIT with C5aAP (YSFKPMPLaR) resulted in two- to five-fold better LS174T xenografts responses than RIT alone. This therapeutic improvement in colorectal adenocarcinoma model was primarily attributed to the agonist-induced increase in the tumor vascular permeability (Kurizaki *et al.*, 2002; Kurizaki *et al.*, 2004). In the current study, the effects of C5aAP and other peptides able to change vascular permeability of tumor blood vessels of radioimmunotherapy of prostate cancer are evaluated.

Summary of the Statement of Work Progress

Objective 1: Determination of the uptake of ^{125}I-labeled B72.3 monoclonal antibody in three human prostate adenocarcinoma models: PC-3, DU-145 and LNCaP in athymic mice. In this Objective the following specific tasks are included:

1. In vitro culture of PC-3, DU-145, LNCaP for implantation into mice: *months 1 – 36*
   In progress
   This is an ongoing task. Because TAG-72 antigen is only expressed in tumors grown in vivo, a constant supply of large number of cultured cells for implantation into mice is needed.
2. Radiolabeling of B72.3 with ^{131}I, ^{125}I, ^{90}Y, and ^{111}In. *months 1 – 36*
   In progress
   This is also an ongoing task. All biodistribution and imaging studies are done with ^{125}I- and ^{111}In-labeled antibodies. For therapy studies we are using ^{131}I- and ^{90}Y-labeled antibodies (also used in Objective 4).
3. Immunohistochemistry to determine TAG-72 expression *months 1 –6*
   Completed: reported in the progress for the first year of funding.

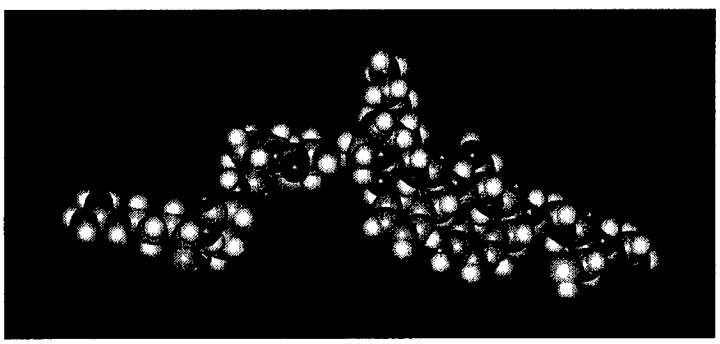

Figure 1. (GCG)-YSFKPMPLaR
Response selective C5a complement agonist

Additional in vitro studies were conducted during the second and third year of funding to evaluate the potential of new C5aAP peptide in RIT (Peptide N2). This new peptide was prepared for two reasons: (1) to allow chemical modifications such as attaching the peptide to large molecular weight carriers (dendrimers; p-Lys) as well as peptide modification with ligands to form complexes with radiometals such as ^{90}Y and ^{111}In; (2) in its unmodified form, i.e., with a free thiol group, to be used in the evaluation of the role of free radicals/reactive oxygen species on RIT. The structure of the new peptide is shown in Fig.1. Three amino

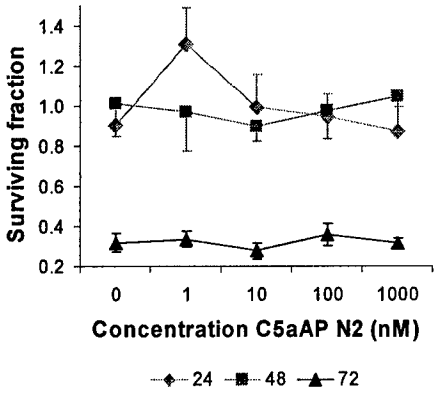

Figure 2. Survival curves of LNCaP cells treated with various concentrations of the C5aAP peptide N2 and irradiated at 6 Gy. Surviving fractions were calculated as a ratio of OD of 6-Gy-irradiated cells to not irradiated cells.

acids, GCG, were added at the NH_2 terminus of the original YSFKPMPLaR peptide (peptide N1). The in vivo effects of this new peptide on the VP were virtually indistinguishable from these of the peptide N1. However, the in vitro studies indicated that N2 is able to attenuate effects of radiation on cell survival up to 48 hours post irradiation (Fig. 2; blue squares). LNCaP cells were grown for 24 h in the presence of varying concentrations of N2 and were either irradiated or not (control) to determine the effect of N2 on the repair/proliferation and the free radical scavenging. Cell survival was measured 24 h, 48h and 72 h after irradiation. For the in vitro cell growth assay, cells were seeded in 96-well plates at a density of 3,000 cells per well in either full growth medium (Eagle Minimum essential medium with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, supplemented with 10% fetal bovine serum) or serum-depleted growth medium containing 0.1% bovine albumin. After 24 h of growth, the culture medium was replaced with fresh medium containing indicated in Fig. 2 concentrations of the N2 peptide and the cells were allowed to grow for 24 h, 48 h, and 72 h; n=6 wells per concentration and time point. Subsequently, a colorimetric assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI) was used to measure the metabolic activity of cells. The fractional growth of not irradiated control cells was considered to be one at each of the N2 concentrations. The in vitro radiosensitization assay was performed as described above with the addition of irradiation of the cells after the 24 h incubation with C5aAP at two doses: 1 Gy or 6 Gy at 1.9 Gy/min in the Mark I 68A research irradiator (6,000 Ci Cesium-137 source, J.L. Shepherd and Associates, San Fernando, CA). Cells were allowed to grow for 48 h before the cell proliferation was determined using a colorimetric kit (Promega). Identical assays were also conducted with DU145 and PC-3 cells. The results were nearly identical. At 24 h post irradiation, the N2 peptide appears to have chemotactic effect on the cell's metabolic activities particularly at lower concentrations. This effect is apparent only in the irradiated cells and is absent when cell survival is measured at 48 h and 72 h (Fig. 2). The evaluation of the cell growth at 48 h after irradiation indicates that N2 has either a protective effect on irradiated cells or stimulates proliferation/repair that is read in this particular assay as increased survival. This effect is lost at later time points after irradiation. Additional tests are in progress using clonogenic assay and the N2 peptide modified at the thiol group to verify the nature of this protective activity.

Objective 2: To measure the effect of C5aAP on the uptake of ^{125}I-labeled B72.3 monoclonal antibody in a prostate cancer model selected in Aim 1 for the expression of TAG-72 antigen. In this Objective the following specific tasks were planned:

1. Synthesis of peptides *months 12 –36*

In progress

This was an ongoing task in years 2 and 3 of this project. Initial studies were done with the previously used C5aAP N1 analog. Two new peptides are being evaluated in search of more response-selective derivatives.

2. Biodistribution of ^{125}IB72.3 in tumor-bearing mice *months 1 –36*

In progress

The biodistribution of the antibody is established in the selected tumor models. We are continuing to evaluate the effects of peptide dose and dosing schedule on the metabolic fate of radiolabeled antibodies. This is done either via a terminal procedures, i.e., necropsy of tumor-bearing animals as well as via imaging studies.

2. MRI of vascular permeability in tumor-bearing mice *months 12 –36*

In progress

The initial results on tumor perfusion and water content with and without contrast were reported in the progress report for year 1 and 2. Further efforts concentrated on the effect of peptides on tumor vessel permeability, blood flow and the transport of macromolecules. These were evaluated using contrast agents with different molecular weights such as DTPA conjugated to IgG or albumin and labeled with a mixture of ^{153}Gd and non-radioactive Gd isotope. The development of data analyses to provide quantitative data on the tumor vessel permeability is still in progress. At this point it is apparent that this technique will not bring the desired level of information in a mouse as a model. The diameter of the blood vessels is very small in mice and the unambiguous identification of these vessels within tumor is extremely difficult rendering our attempts to obtain quantitative evaluation of vessel permeability ineffective. The efforts were therefore shifted to the SPECT evaluation of radiotracer uptake and the homogeneity of distribution within the tumor as markers of the altered vascular permeability.

Objective 3: To conduct experimental therapy studies with ^{131}I-B72.3 RIT and in combination ^{131}I-B72.3 with peptide in human prostate cancer model.

All efforts in this Objective are concerned with the therapeutic studies and the following specific tasks are associated with this Objective:

1. RIT of prostate cancer in tumor-bearing mice *months 12 –36*

In progress

This was an ongoing task in years 2 and 3 of this project and overlaps with the task 2 in this Objective. The tumor responses to augmented RIT were significantly improved and require much longer follow up times. On this basis, we have requested a no-cost extension until Nov. 30, 2005 to finalize all therapy studies and to publish the data. Our request was granted on Nov. 16, 2004.

2. Biodistribution and termination of therapy protocols *months 12 –36*

In progress

All therapy protocols are terminated when the tumor size in control groups reaches about 10% total body weight (as per instructions of the UNMC IACUC). We continue to evaluate antibody retention and tumor vascular changes in necropsied tumor samples. When possible, i.e., sufficient recovery of radiolabeled material in blood, a determination of the immunoreactivity of recovered radioactive species and levels of TAG-72 in treated and non-treated tumors is also performed.

Objective 4: Comparison of the efficacy of ^{131}I-labeled versus ^{90}Y-antibodies in peptide-augmented RIT protocols.

These studies just started. We have completed two ^{90}Y-therapy protocols in mice bearing PC-3 tumors. The LNCaP and DU-145 studies will begin in the next few months. The experimental evidence from carcinoma xenografts indicates that ^{90}Y may be a superior choice as a therapeutic radioisotope. To date, there were no studies to confirm this in prostate cancer models. There are also no studies on the effect of biological response modifiers on the outcome of ^{90}Y-RIT. We are in the process of investigating these questions. A significant concern emerged from these early stage studies, i.e., the health of mice treated with doses of ^{90}Y as low as 0.25 mCi rapidly deteriorates because as a result of a longer retention of the radioisotope in some organs. Attempts to improve this situation are described below in the Results section.

Results

Effects of C5aAP on RIT tumor distribution

Mice were treated with either PBS or C5aAP for up to five consecutive days. The peptide was injected IV via a tail vein at the dose of 10 mg/kg. In all studies ^{125}ICC49 was given IV always 3 h after the dose of the peptide. Additional doses of C5aAP were given IV every 24 hours. Tumors were immediately snapped frozen. Alternating sections were uses for either macroautoradiography or microautoradiography. The image analysis was done using the ImageJ software (ImageJ is a public domain image analysis program that was developed at the National Institutes of Health; http://rsb.info.nih.gov/ij/; accessed on Dec. 20, 2004).

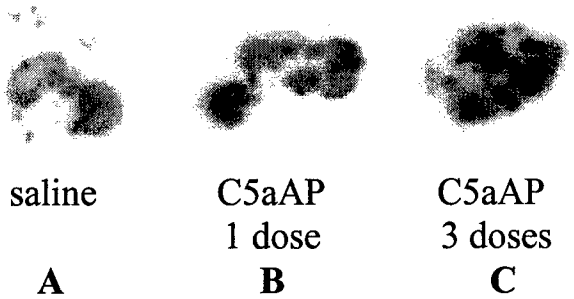

Figure 3. Macroautoradiography of 5-μ center sections of PC-3 human prostate adenocarcinoma xenografts excised from athymic treated with either saline (A) or 10 mg/kg QD C5aAP peptide (B and C) and a dose of ^{125}ICC49 three hours later. Mice were euthanized 72 h post injection. Sections of tumors were placed directly on a Kodak AR film, stored at -80°C for 24 h and developed. Dark areas correspond to radiolabeled antibody.

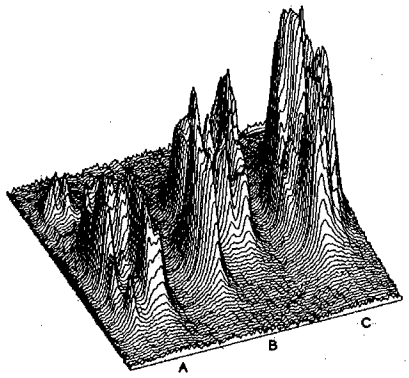

Figure 4. Surface plot analyses of the macroautoradiography images shown in Fig. 3. Not only the absolute amount of radioactivity accumulated in tumor increases with the administration of C5aAP but the homogeneity and consequently the distribution of radiation dose is much improved.

Fig. 3 shows a significantly improved uptake and homogeneity of the radiotracer within the PC-3 xenografts unmistakably indicating that in response to the C5aAP-induced VP changes, the influx of the radiotracer into the tumor is better and its distribution much more homogenous. Tumors were frozen immediately after resections; 5-μ sections were prepared, placed on the X-ray film and films were exposed for 24 h. The tumor sections were counted in a specially designed scintillation counter and after the developed films were analyzed, the radioactivity (cpm) was correlated with the pixels in analyzed images. The plot analyses in Fig. 4 confirmed

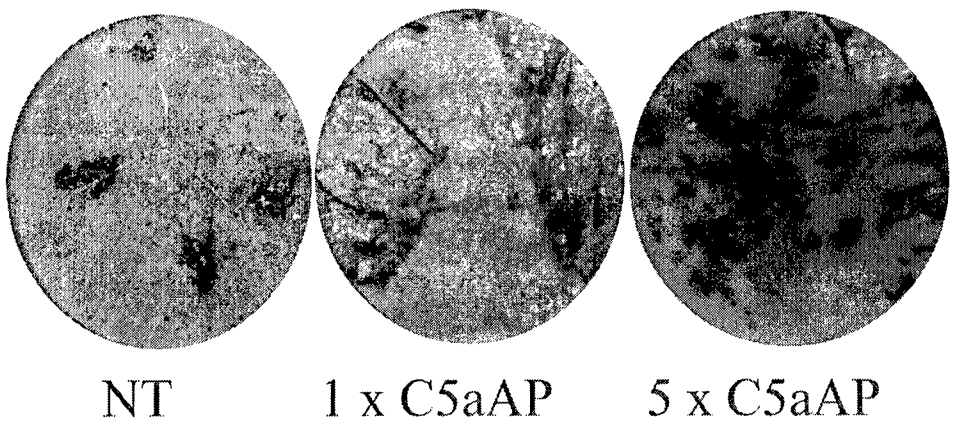

Figure 5. Microautoradiography of section of PC-3 xenografts from mice treated with either saline or C5aAP peptide and ^{125}ICC49. The fixed sections were dipped in emulsion, decays were accumulated for 3 days, and slides were developed. Original magnification x10; counterstained with H&E. Radiolabeled is detected as silver grains (black) in sections above.

our gross observations. These C5aAP-induced changes are also verified on the microscopic level (Fig. 5). ^{125}ICC49 in control tumors treated with saline in place of C5aAP show heterogeneous accumulation of radioactivity near the blood vessels. The addition of a single dose of C5aAP improved the radioisotope distribution and tumors treated with five doses C5aAP are just about uniformly labeled.

Imaging studies of vascular permeability effects on RIT tumor distribution

The data acquired in noninvasive SPECT imaging studies parallel the macro- and microscopic results shown above (Fig. 6). Images collected 24 hours after the administration of ^{125}ICC49 in control mice show tumor uptake of the radiotracer, however, the amounts are low compared with images after one or two doses of

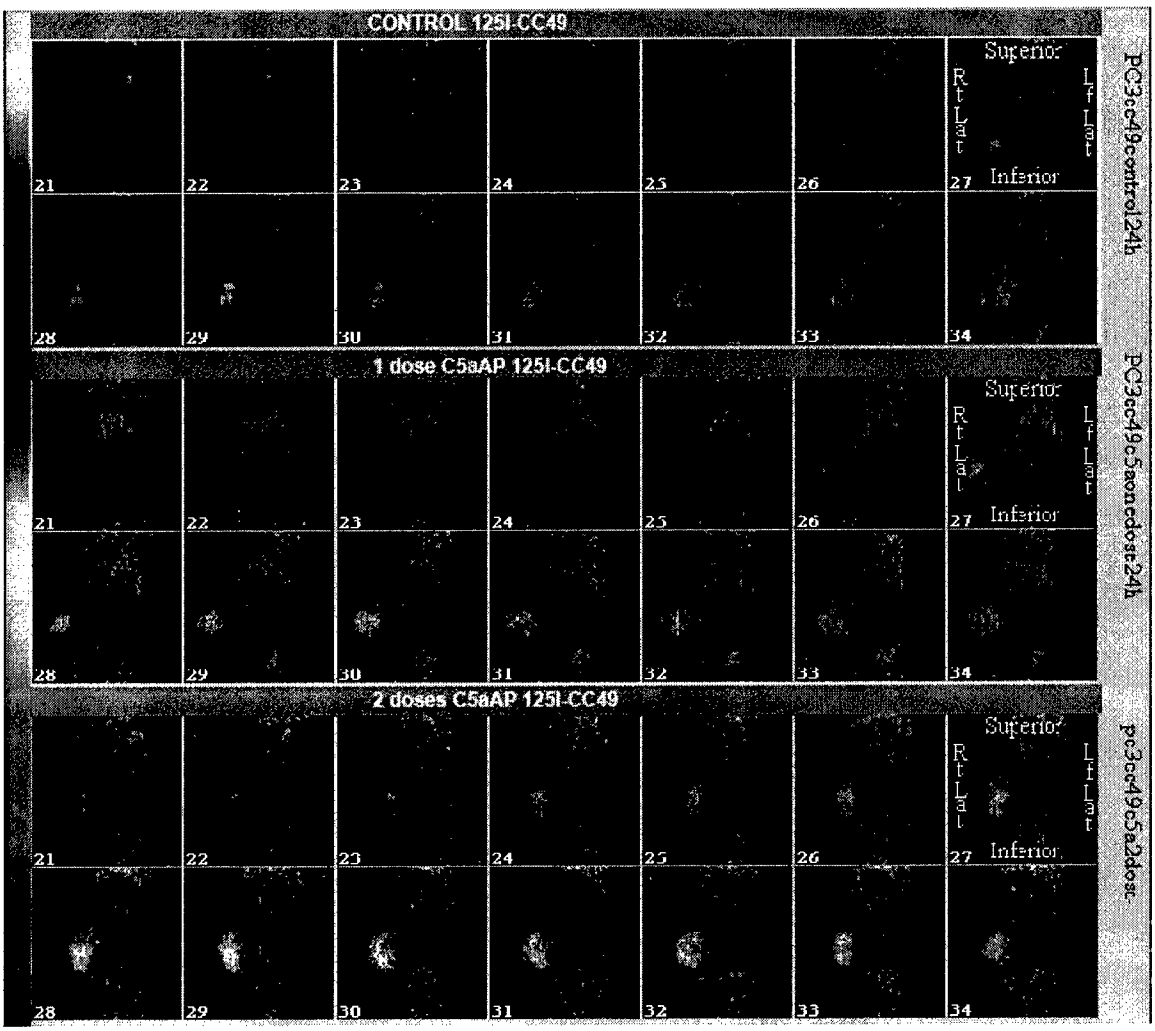

Figure 6. SPECT images acquired 24 hours after administration of 0.25 mCi ^{125}ICC49. Tumors are on the left side. There is significant improvement in the uptake of ^{125}ICC49 in mice treated with C5aAP.

C5aAP. The bar in Fig. 6 is a scale of the intensity of radioactivity uptake. The red color is a maximum, the blue color is minimum, and the black is the background radioactivity. The SPECT analysis of the center sections of the PC-3 tumors is shown in Fig. 7. Similarly to the autoradiography results, the C5aAP-treated tumor have higher and more uniform uptake of the radiotracer. For imaging studies ten mice with size-matched PC3 tumors (range 0.44 g - 0.71 g) were selected from groups treated with either C5aAP (10 mg QD; n = 8) or saline (n = 2; control). ^{125}ICC49 (0.25 mCi) was injected intravenously and the imaging commenced 24 h, 48, or 72 h after the administration of the radioactive tracer. Images were acquired using a dedicated Animal SPECT Imaging

System (Gamma Medica Instruments, Northridge, CA). The image was reconstructed using LumaGEM version 5.107 software with the Butterworth bandpass post-reconstruction filtering. To obtain quantitative evaluation of the uptake, the total counts in the region of interest drawn around the perimeter of the tumor were measured and divided by the number of pixels for each tumor at each time point and the tumor-specific uptake was obtained after subtraction of the background counts.

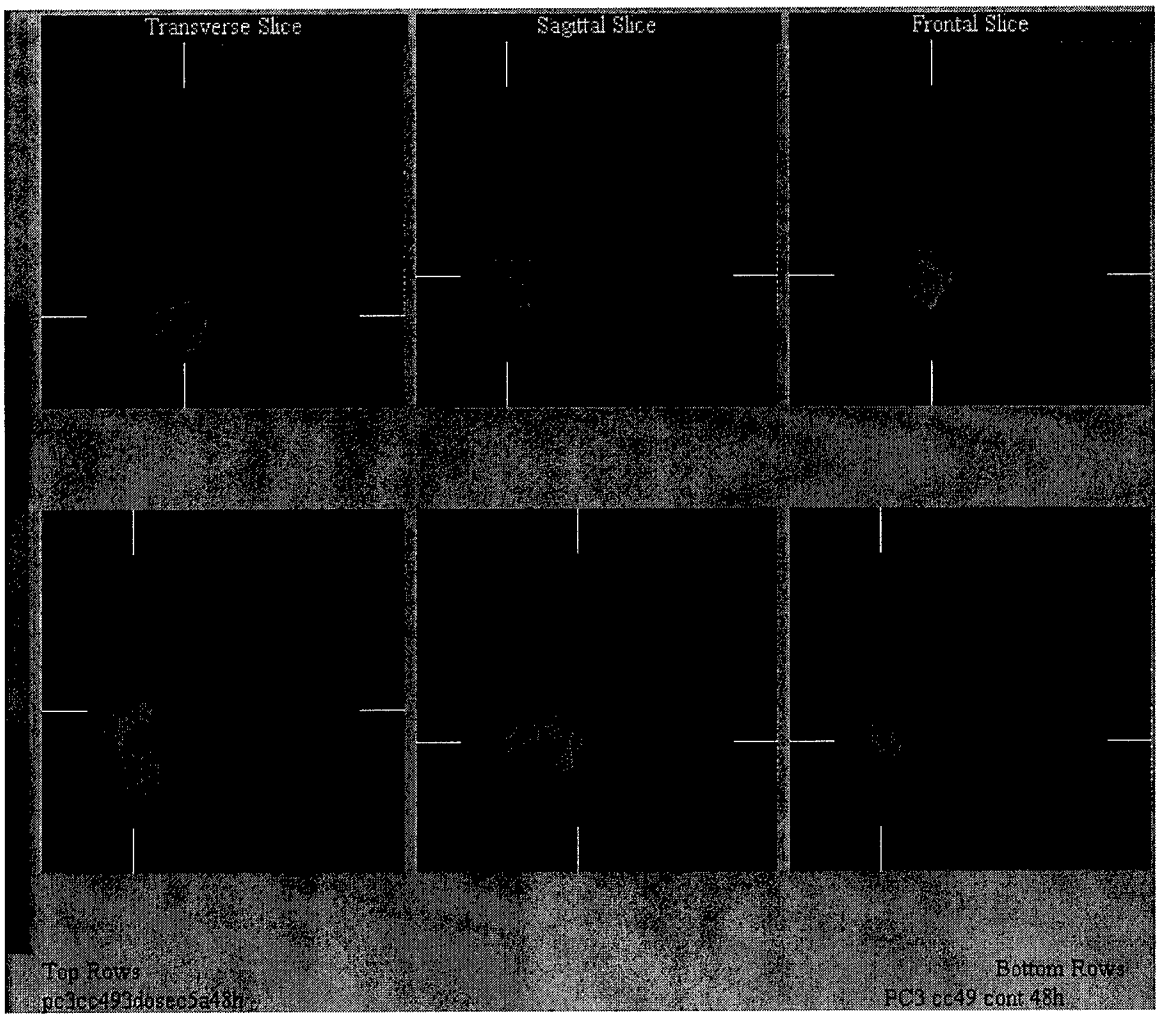

Figure 7. Transverse, sagittal and frontal images of a center slice of PC3 prostate adenocarcinoma grown as subcutaneous xenografts in athymic nu/nu mice. Images were acquired 48 h after the administration of 0.25 mCi ^{125}ICC49. The three upper panels are images of a mouse treated with 3 doses of C5aAP (10 mg/kg QD). The lower panels are images of a mouse treated with sham injections of saline. The panel on the left side of the images is a scale of radiotracer uptake. The dark red color corresponds to the highest levels of radioactivity; black is the color of background levels of radioactivity.

Biodistribution of imaging mice treated with either PBS or C5aAP was conducted 72 h after ^{125}ICC49 administration, tumors were removed, and the amount of radioactivity in tumor, blood and selected tissues was measured. The uptake was expressed as percent injected dose per gram tumor. Fig. 8 shows the absolute uptake expressed in mCi/g tumor as well as the tumor-to-liver ratios in the same animals. It appears that the inclusion of the peptide in RIT may have additional benefits, i.e., the rate of clearance from normal tissues seems to be enhanced (Fig. 8, panel B).

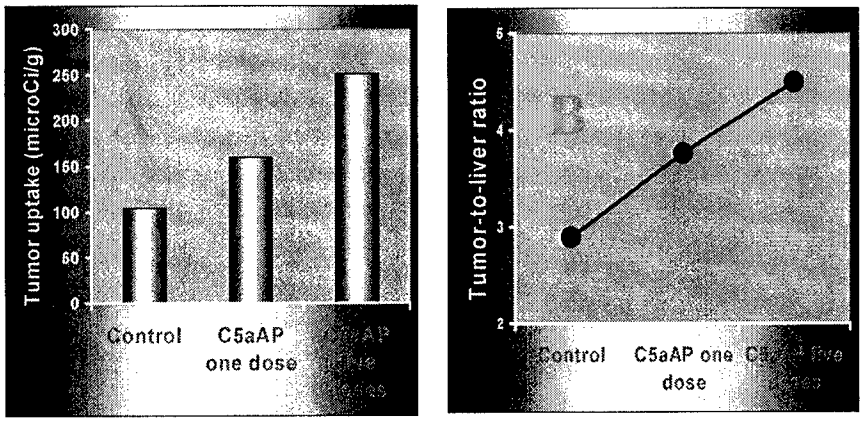

Figure 8. Tumor uptake of ^{125}ICC49 in PC3 xenografts after treatment with various doses of C5aAP or saline (controls). Five doses of C5aAP increase tumor uptake by >150% (panel A). In addition clearance from normal tissues also seems to be improved as shown in panel B for tumor-to-liver ratios.

The improved uptake and distribution of radiolabeled antibodies translated into a much improved tumor response to RIT. For RIT studies mice received a SQ implant of 2×10^6 PC-3 cells in matrigel. Forty days later when the average tumor size was >300 mm^3 mice were randomized into four groups as follows: *group A*: no treatment (n = 10); *group B*: ^{131}ICC49 only (n = 10); *group C:* C5aAP only (n = 12); and *group D*: ^{131}ICC49 plus C5aAP (n = 20). Body weight and tumor sizes were measured three times a week, and tumor volumes calculated according to the following formula: [(π×longer diameter × (shorter diameter)^2)/6]. Mice in group D received C5aAP IV via a tail vein at 10 mg/kg dose in PBS and ^{131}ICC49 (0.25 mCi) also IV via a tail vein in 0.2 mL PBS 3 hours later. Group B control mice received either saline in place of C5aAP and 3 hours later ^{131}ICC49 (0.25 mCi) also IV via a tail vein in 0.2 mL PBS; mice in group C were treated with 10 mg/kg C5aAP and 3 h later with a sham injection of saline in place of radiolabeled antibody; and finally group A untreated controls received two IV injections of slaine according to the same schedule. Forty eight hours before termination of the experiment, all mice were received an IV dose of 50 μCi ^{125}IUdR to enable measurement of the tumor proliferative rate. Excised tumors were lysed and the amount of ^{125}IUdR bound to DNA was determined using the DNA Extractor WB Kit - Sodium Iodide method (Wako Chemicals USA, Inc, Richmond, VA). The experiment was terminated after six weeks when the tumor sizes exceeded 10% body weight. The Kaplan-Meier analyses were used to determine survival using the tumor quadrupling time as the surrogate end point. Fig. 9 shows a typical outcome for PC3 tumors. The combination RIT+C5aAP even at low 0.25 mCi doses of ^{131}ICC49 produces very favorable outcome (Table 1).

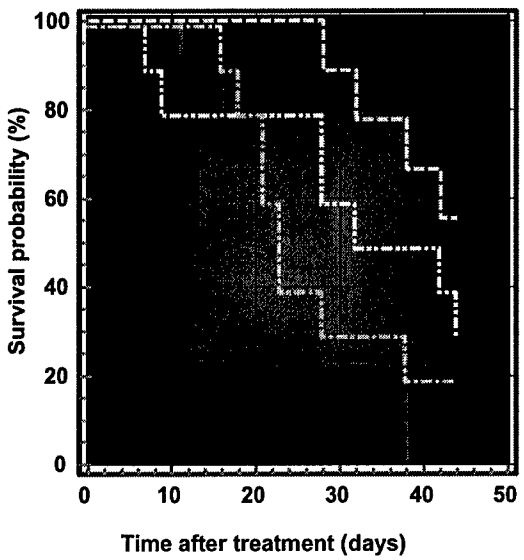

Figure 9. Kaplan-Meier analyses of tumor quadrupling time (survival) in mice bearing PC3 human adenocarcinoma xenografts treated with 0.25 mCi ^{131}ICC49 and one 10 mg/kg dose of C5aAP (cyan dashed line); 0.25 mCi ^{131}ICC39 (light green two dots-dashed line); one dose 10 mg/kg C5aAP (blue one dot-dashed line); and PBS treated controls (navy dashed line).

Table 1. Kaplan-Meier estimates of median survival based of the tumor quadrupling time as a surrogate time point for survival.

treatment	sample size	median survival (days)
^{131}ICC49 + C5aAP	20	not reached
^{131}ICC49	10	37
C5aAP	12	23
saline	10	23

Chi-square = *8.9448*
Significance *P = 0.0300*

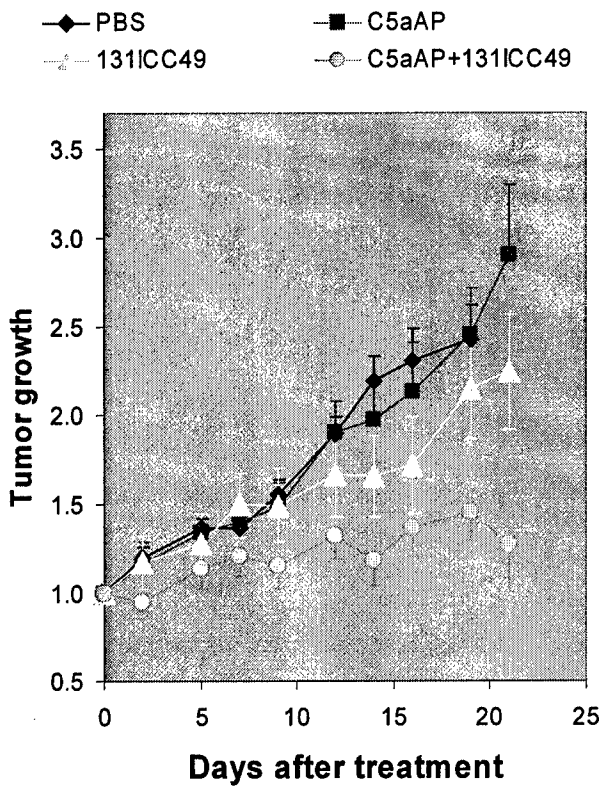

Figure 10. Growth rate of DU-145 human prostate adenocarcinoma xenografted in athymic nu/nu mice treated with C5aAP and ^{131}ICC49 (cyan circles) as compared to controls treated with either ^{131}ICC49 alone (yellow triangles), C5aAP alone (purple squares), or saline (navy dimonds).

augmented RIT regimens brought a degree of disappointment. Even though there were improvements in tumor responses (Fig. 11), there was very unfavorable effect of 90Y on the overall health of mice that prompted the termination of the studies 14 days after the RIT administration. The rapid body weight losses and some intestinal distress were all attributed to the radiation damage of normal tissues. ^{90}Y deposits all of its decay energy in a relatively small volume resulting in large radiation doses to normal organs; the path length for ^{90}Y χ_{90} = 5.2 mm compared to 0.7 mm for ^{131}I (χ_{90} is the distance within which 90% of the energy is deposited within the radius). In our studies much like in the clinical protocols, we found that the dose–limiting toxicity was related to the bone marrow suppression and resulting cytopenias.

Kaplan-Meier survival analyses were done using MedCalc Software Ver. 7.4.4.0 (Mariakerke, Belgium). To assess differences in tumor growth between treatment groups the generalized estimating equations were used. The logrank test for trend analyses of tumor growth was done using the GraphPad InStat version 3.00 for Windows 95, (GraphPad Software, San Diego California).

Similar outcome was also observed in LNCaP and DU-145 models (some of these data were already reported for a single dose C5aAP studies). The effect of multiple doses of C5aAP on the tumor growth is more then additive, i.e., two 0.1 mg/mouse doses of C5aAP in 24 h intervals plus 0.25 mCi ^{131}ICC49 reduce tumor growth reduction more effectively then one 0.2 mg/mouse dose of C5aAP and 0.25 mCi ^{131}ICC49 (Fig. 10). The data analyses are shown in Table 2 below. It should be noted that of the three tumor models tested in these studies, DU-145 seem to have the lowest expression of the antigen TAG-72 as based on the biodistribution studies. Despite of this problem, the responses to the combination RIT+C5aAP are impressive. The experiment was terminated three weeks after the first dose of C5aAP because tumor sizes in control groups exceeded the allowed dimensions.

Two ongoing studies will compare the outcome of RIT combined with C5aAP in a multiple dose regimen to RIT alone. These will be followed by a similar therapy regimen with the WKYMVm peptide in place of C5aAP.
The comparison of ^{90}Y-labeled antibodies in the

Table 2. T-test analysis of the DU-145 growth in athymic mice treated with C5aAP and ^{131}ICC49 compared to controls.

	day 7	**day 19**
PBS versus C5aAP	0.8275	0.9364
PBS versus ^{131}ICC49	0.5404	0.4293
PBS versus ^{131}ICC49+C5aAP	0.3356	**0.0011**
C5aAP versus ^{131}ICC49	0.6800	0.4708
C5aAP versus ^{131}ICC49+c5aAP	0.2523	**0.0069**
^{131}ICC49 versus ^{131}ICC49+c5aAP	0.1953	**0.0427**

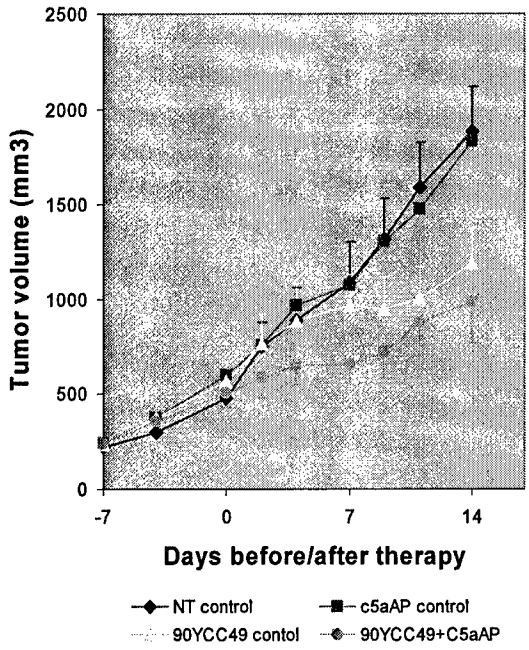

Figure 11. Tumor sizes in mice treated with ^{90}Y-DOTA-CC49 with or without the addition of C5aAP. The study was terminated two weeks after the administration of 0.25 mCi ^{90}Y-DOTA-CC49 because of the rapid weight loss in ^{90}Y-treated mice.

The experimental design for these studies parallel other therapy experiments. Mice were injected with 2×10^6 tumor cells in matrigel. Six weeks later therapy mice were treated with 10 mg/kg C5aAP followed 3 h later by an IV dose of 0.25 mCi ^{90}Y-DOTA-CC49. Controls received according to the same schedule either saline + ^{90}Y-DOTA-CC49; or C5aAP + saline; or two doses of saline. Mice were evaluated every other day at the first signs of distress the experiment was terminated.

Two modifications were introduced to the ^{90}Y RIT protocols: (1) the pretargeting strategy was employed (ongoing), whereby the C5aAP was used in conjunction with unlabeled biotinylated CC49. This approach allows a better accumulation of CC49 in tumor followed 72 h later by administration of avidin and finally 24 h after the avidin ^{90}Y-DOTA-biotin as the radiotherapeutic (this study is ongoing). Using pretargeting methods allowed us to take advantage of the C5aAP effects and localize large quantities of biotinylated CC49 (not radioactive and therefore not dangerous to animal well being). Further advantage comes from the fact that because each avidin molecule can bind four biotin molecules, the ^{90}Y activity in tumor can be further increased. This is an ongoing study and therefore it is premature to speculate on the final outcome. The initial indication is that four weeks into the therapy, the body weights and overall health of mice in the treatment group is identical to saline-treated controls.

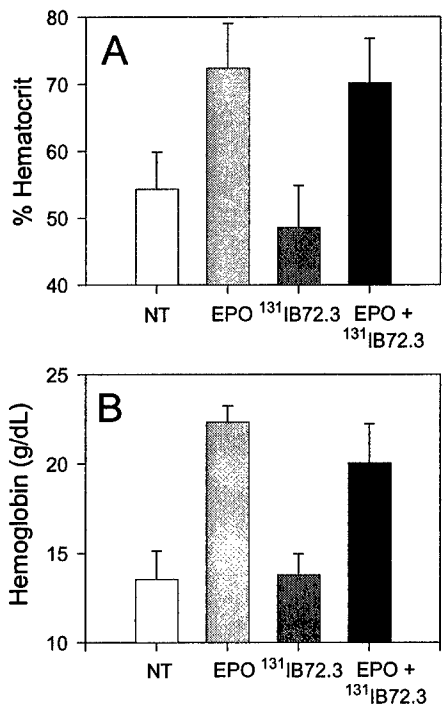

Figure 12. Levels of hematocrit (panel A) and hemoglobin (panel B) in response to the erythropoietin treatment in LS 174T-bearing athymic mice. **A.** *P=0.002* for untreated controls versus Epo-treated mice; and *P=8.7E-06* for ^{131}IB72.3- versus ^{131}IB72.3+Epo-treated mice. **B.** *P=4.7E-06* for untreated controls versus Epo-treated mice; and *P=1.6E-03* for ^{131}IB72.3-treated versus ^{131}IB72.3 plus Epo treated mice.

Table 3. Tumor doubling times and tumor sizes in control mice treated with sham injections of phosphate buffered saline and mice treated with Epo.

Treatment	Doubling time days ± std dev	*P values* doubling times	Initial tumor size mm^3 ± std dev	*P values* tumor size
no treatment control	3.41 ± 0.35	P = 0.012*	56 ± 29	P = 0.840†
EPO	2.74 ± 0.61		50 ± 31	
no treatment control	6.36 ± 1.75	P = 0.052*	297 ± 125	P = 0.626†
EPO	4.86 ± 0.96		285 ± 110	

* *Mann-Whitney Rank Sum Test*
† *t-test*

The second approach involved the inclusion of erythropoietin in the RIT scheme to ease the unfavorable effects of cytopenia. Erythropoietin (Epo) is an established treatment in the management of patients with chronic renal disease. In these patients, Epo has been shown to improve hemoglobin levels, decrease red blood cell transfusion requirements, and improve quality of life (Eschbach et al, 1988). Epo is also recommended as a safe and effective treatment to reduce the incidence of symptomatic treatment-related anemia and the need for the blood transfusions in the management of cancer patients with non-hematological malignancies receiving chemotherapy and radiotherapy. The clinical evidence links decreased hemoglobin levels with inadequate oxygenation of solid tumors and in a recent study, Becker et al. (2000) demonstrated that severe anemia significantly lowers the tumor pO_2 and produces high tumor hypoxic fractions in patients with head and neck cancers. Several authors hypothesized that the blood in anemic patients carries less oxygen and consequently this leads to the decreased arterial oxygen supply to the tumor and the reduction of the therapeutic effect of radiotherapy. For example, Stuben et al. (2003) used recombinant human Epo to correct radiation-induced anemia in mice and to investigate the consequences of this anemia correction on tumor oxygenation and the efficacy of radiotherapy. Based on these data, we hypothesized that the maintenance of the red blood cell volume throughout the treatment may represent a vital pathway not only to improved animal health but also to the tumor radiosensitivity. We further hypothesized that because the decreased oxygen supply to the tumor has an unfavorable effect on tumor responses to RIT, the inclusion of Epo in the RIT regimen should improve tumor responses by reversing tumor hypoxia. The pilot study was done in LS1 174T tumors because this model has a more favorable expression of the antigen and because the rapid growth of LS 174T tumors. Mice were randomized into four groups: (1) no treatment (n = 16); (2) ^{131}IB72.3 only (n = 18); (3)

Table 4. Median survival times and *P* values for Kaplan – Meier tumor quadrupling time analyses two weeks after treatment with 0.25 mCi ^{131}IB72.3.

Treatment	**Number of mice**	**Median survival**	**P values**
NT control vs EPO	16	NT = 10 d	0.0038
EPO vs ^{131}IB72.3	18	EPO = 6 d	0.0003
EPO vs EPO+^{131}IB72.3	18	^{131}IB72.3 = 15 d	0.00001
^{131}IB72.3 vs EPO+^{131}IB72.3	18	EPO+^{131}IB72.3 = 17 d	0.6884

Epo only (n = 18); and (4) ^{131}IB72.3 plus Epo (n = 18). Body weight and tumor sizes were measured three times a week. Epo was diluted in sterile water to a final concentration of 2,000 U/mL and injected subcutaneously at a dose of 2 U/g of body weight on days -3, -2, 0, 1, and 4 before and after RIT. ^{131}IB72.3 (0.25 mCi) was injected IV via a tail vein in 0.2 mL PBS on day 0, 2 h after Epo. Two days before termination of the experiment, all mice received IV 30 μCi ^{125}IUdR to enable the measurement of tumor proliferative rates.

The biodistribution was conducted 48 h later. Blood, liver, spleen, kidney, and tumor were collected and their radioactive content determined in a multichannel NaI γ-counter (Minaxi, Packard Instruments). For hematocrit measurements blood was collected by direct cardiac puncture immediately before death into a heparinized syringe, 0.1 mL aliquots of heparinized blood were placed in hematocrit capillary tubes, spun for 5 min at 500 x g and hematocrit (%) was determined by standard techniques. The HemoCue® (Ängelholm, Sweden) method was used to measure hemoglobin levels. A drop of fresh blood was placed in a HemoCue® microcuvette and the levels of hemoglobin were read at 570 and 880 nm according to the manufacturer's instructions. The instrument was calibrated daily.

The Epo effects on anemia of tumor-bearing mice were practically as predicted from these already reported in mice and human. The hematocrit and hemoglobin levels as well as body weights of the Epo-treated mice enrolled in the RIT protocol were much improved compared to the Epo-untreated controls (Fig. 12). These are in accord with the observations made by others (e.g., Stuben et al, 2003). However, this is were the similarities between ours and cited above studies end. Unlike the radiation therapy of human glioma xenografts that benefited from the inclusion of Epo, subcutaneous LS 174T xenografts showed an astonishing acceleration of growth in mice receiving Epo. The rate of growth was greatly dependent on the timing of the Epo treatment. Smaller tumors (<100 mm^3) profited from the Epo inclusion more effectively. In twelve days, tumor sizes increased by >170% compared to tumors that were not treated with Epo. In this group of mice, the T_D of Epo-treated tumors decreased from 3.41 ± 0.35 days to 2.74 ± 0.61 days (Table 3). This effect was less dramatic when Epo treatment was commenced when tumors reached the size >200mm^3 and the T_D values were also affected to a lesser degree (Table 3).

Tumor responses to RIT in mice treated with Epo were not better than tumor responses to ^{131}IB72.3 alone. Any benefits from a better health of Epo-treated mice or the improved tumor oxygenation were severely offset by the accelerated tumor growth when treated with Epo. The RIT-induced tumor growth delay (T_D = 12.05 ± 4.89 d for ^{131}IB72.3 alone) was not significantly different from the effect of Epo-augmented RIT (T_D = 11.22 ± 2.59 d; *P = 0.5853*). There was a slight but not statistically significant improvement in the median survival of the Epo+^{131}IB72.3-treated mice (Table 4). The effect of Epo on the proliferative rate of xenografts as measured by the ^{125}IUdR uptake was consistent with the accelerated tumor growth. The tumor-to-blood ratio of ^{125}IUdR in mice treated with ^{131}IB72.3 and Epo was four times greater than in mice treated with ^{131}IB72.3 only. The proliferative factors were 3.6, 6.2, 1.3, and 5.0 for untreated controls, Epo-, 131IB72.3, and 131IB72.3 + Epo-treated mice, respectively. Although the overall outcome of the Epo-augmented RIT is disappointing, the tumor growth delay despite the Epo-accelerated tumor proliferation in mice treated with ^{131}IB72.3+Epo is a good indication that the radiosensitivity of Epo-treated xenografts is appreciably improved probably in response to the enhanced tumor oxygenation.

Key Research Accomplishments to Dates

- ✓ The advantages of the RIT augmented with vasoactive peptides was confirmed for three peptides: WKYMVm and two C5aAPs and proved to work in all three prostate adenocarcinoma models: LNCaP; DU145 and PC3.
- ✓ Determined the expression of C5a receptors and effect of a peptide agonist of human C5a complement on the metabolic activities of in vitro grown prostate adenocarcinoma cells.
- ✓ Functions of the C5aAP peptides in RIT and the basic mechanism of their action were established and the C5aAP- or WKYMVm-induced production of reactive oxygen species/free radicals was identified as an

additional factor in the improvement of the RIT results.

- ✓ New peptide WKYMVm that increases vascular permeability and generates superoxide was used in a RIT protocol and was shown to greatly improve the outcome of RIT indicating that the synergy/additive effects observed with C5aAP may be at least partially a result of the interaction of radiation with in situ generated reactive oxygen species.
- ✓ Using noninvasive SPECT and MRI techniques, determined the C5aAP-induced changes in tumor perfusion in the experimental model of human prostate adenocarcinoma and correlated these with a better tumor uptake of RIT and a more homogenous distribution of radioactivity.
- ✓ These noninvasive methods were used to confirm typically used data generated in the biodistribuion studies.

Reportable Outcomes

Kurizaki T, Abe M, Sanderson SD, Enke CA, Baranowska-Kortylewicz J. Role of polymorphonuclear leukocytes, nitric oxide synthase, and cyclooxygenase in vascular permeability changes induced by C5a agonist peptides. Mol Cancer Ther. 2004 Jan;3(1):85-91.

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Abe M, Nearman J, Kortylewicz ZP, Boska M, Pavlinkova G, Mosley RL, Baranowska-Kortylewicz J. CD88-Dependent methods to improve radioimmunotherapy of prostate cancer. EOARTC-AACR-NCI International Conference. Molecular Targets and Cancer Therapeutics: Discovery, Biology, and Clinical Applications. Geneva, Oct. 2004.

We are also preparing four manuscripts that detail the effects of C5aAP and WKYMVm on RIT. However, because several therapy protocols are still in progress, these will not be submitted until all data is finalized.

Conclusion

- ✓ The inclusion of C5aAP or WKYMVm in RIT improves the outcome of RIT of the experimental prostate adenocarcinoma grown as subcutaneous xenografts in athymic mice.
- ✓ The responses to ^{90}Y-RIT + vasoactive peptides are impaired because of the radiation side effects.
- ✓ C5a Receptors (CD88) are expressed on some prostate cancer cells and may have an effect on the tumor growth and response to RIT.
- ✓ Increased levels of tumor oxygenation in response to stimulation of neutrophils with peptides appear to radiosensitize tumors to radioimmunotherapy.
- ✓ Addition of erythropoietin to improve animal health during RIT accelerates tumor growth in one model studied to date.
- ✓ Pretargeting RIT strategies will benefit from the inclusion of vasoactive peptides (ongoing).

Abbreviations:

C5aAP	response-selective peptide agonist on the human C5a complement
CC49	second generation monoclonal antibody that recognizes TAG72 in most of human adenocarcinomas
B72.3	precursor monoclonal antibody of CC49
nM	concentration: nanomole/liter

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